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Purification and Characterization of Intrauterine Folate-Binding Proteins from Swine[1]

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ABSTRACT

Folate-binding proteins (FBP) from Day 60 pseudopregnant uterine flushings and Day 60 allantoic fluid were purified by affinity chromatography on folate-Sepharose followed by G–100 Sephadex chromatography. FBP from uterine flushings had a molecular weight of 20 000; the N-terminal sequence was FNWDHXGKMEPAXKRHFXXXTXLYX, which is 72% identical to bovine milk FBP beginning at amino acid 64. Allantoic fluid FBP had a molecular weight of 30 000; and the N-terminal sequence ARAKTDMLNVXMDAKHHKPKPSXED, which is 68% identical to bovine milk FBP starting at amino acid 4. Scatchard analysis of purified allantoic fluid FBP using [^{3}H]folic acid as ligand indicated a dissociation constant of 0.54 nM, and the protein was saturated at 20 nM. Antiserum to the purified allantoic fluid FBP was generated in rabbits and used for immunoblotting. Uterine flushings were collected from pregnant and nonpregnant gilts on Days 10, 11, 12, 13, and 15. Immunoblotting indicated that FBP concentrations increased in uterine flushings from both pregnant and nonpregnant gilts between Days 10 and 15. Total uterine flush specific binding of [^{3}H]folic acid increased from 0.015 nmol on Day 10 to 2.14 nmol on Day 15. These results indicate that an FBP similar to other known FBPs is present in uterine flushings and allantoic fluid and that its level increases at about the time of blastocyst elongation and initiation of conceptus hematopoiesis. These results suggest a role for FBP in the delivery of folate to the developing conceptus.

INTRODUCTION

Folic acid is a vitamin that participates in biochemical reactions that transfer methyl groups between substrates [1]. Methionine, purine ring, and thymidylate synthesis all involve folic acid metabolites. Thus, tissues undergoing rapid cell division require folate to maintain the rate of DNA synthesis. Such rapid cell division is characteristic of swine conceptuses during early development and of erythropoietic tissues of swine fetuses during periods when the blood supply of the fetus is being generated. It is likely that mechanisms exist to deliver folic acid to the developing conceptus. However, the mechanism of transport from maternal to fetal compartments in swine has never been elucidated.

Several studies have been performed to determine the influence of folate supplementation on reproductive performance in gilts. Some studies report increased litter size [2–4] while others report no change in litter size [5–7]. Thus, the results of these studies are equivocal. The reason for the inconsistent results may be that increased plasma folate may not result in increased folate delivery into the uterine lumen. Charged molecules, such as folate, would not be expected to diffuse readily across the intact endometrial epithelium of the pig uterus during pregnancy. Instead, a folate-binding activity is present in uterine flushings [8]. It is our hypothesis that this activity is likely secreted by the endometrium and that it transports folate across the epithelial barrier, similar to mechanisms controlling iron (uteroferrin) and retinol (retinol-binding protein) transport [9–16]. Like that for uteroferrin and retinol-binding protein, the rate of iron and retinol delivery to the developing conceptus likely depends on the rate of secretion of the transport proteins. Thus, identification of the protein corresponding to the previously studied folate-binding activity, as well as determination of the manner in which production of that protein is controlled, may allow a more effective means for increasing the folate available to the swine conceptus. The objectives of the present experiments were 1) to purify and characterize folate-binding protein (FBP) in allantoic fluid and uterine flushings of swine and 2) to determine whether the amounts of FBP within the uterus change from Day 10 to 15 of the estrous cycle or pregnancy, a period of rapid growth of the conceptus and initiation of red blood cell synthesis.

MATERIALS AND METHODS

Purification of FBP

All animal-handling procedures were reviewed and approved by our institutional Animal Care Committee. Investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

Approximately 500 ml of pooled uterine flushings from Day 60 pseudopregnant gilts from a previous experiment [17] and 3 L of Day 60 allantoic fluid were collected from white crossbred pregnant gilts. Briefly, each uterine horn of the Day 60 pseudopregnant gilts was flushed with 30 ml Minimum Essential Medium containing one tenth the normal amount of leucine. The flushings were stored at −20°C between experiments and then pooled (18 gilts) to obtain 500 ml. The allantoic fluid was collected at slaughter from 2 gilts. The uterine flushings and allantoic fluid were dialyzed extensively against 50 mM glycine, pH 2.8, to remove endogenously bound folates. Pools were dialyzed against 50 mM Tris, 1 M NaCl, pH 8.2. Then, each pool was passed through a 5-ml column of folic acid-Sepharose (Sigma Chemical Co., St. Louis, MO), and the column was washed with 100 ml of 50 mM Tris, 1 M NaCl. Bound proteins were eluted using 50 mM glycine, 1 M NaCl, pH 2.8. Protein eluting from the column was monitored using absorbance at 280 nm. Samples containing protein were pooled, dialyzed against distilled water, lyophilized, and then subjected to G–100 Sephadex chromatography in 10 mM Tris, 0.33 M NaCl. Initially, 10 μCi [^{3}H]folic acid (specific activity 50 Ci/mmol; Amersham, Arlington Heights, IL) was added before G–100 chromatography to

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determine those fractions that contained FBP, and an aliquot from each fraction was then subjected to scintillation counting. In subsequent runs, nonradioactive folic acid sufficient to saturate the FBP present was added to the sample before chromatography, and elution of proteins was monitored using absorbance at 280 nm. Fractions from G–100 Sephadex chromatography that contained FBP were pooled, dialyzed against distilled water, lyophilized, and then redissolved in a small amount of distilled water. Protein concentration of the purified preparation was determined using the method of Lowry et al. [18]; absorbance at 280 nm was found to dramatically overestimate the amount of protein present. Aliquots of the protein resulting from pseudopregnant flushings and from allantoic fluid were lyophilized and then subjected to SDS-PAGE [19] followed by staining with silver (pseudopregnant flushings) or Coomassie (allantoic fluid) to determine purity and approximate molecular weight. Further aliquots were subjected to SDS-PAGE and then blotted onto polyvinyldifluoride membrane as described by Matsudaira [20]; the major band on the blot was excised and the N-terminal sequence determined (Protein Sequencing Core Facility, University of Nebraska, Lincoln).

Scatchard Analysis

FBP purified from allantoic fluid was used for Scatchard analysis of folate binding. The FBP was stripped of folate by dialysis against 50 mM glycine, pH 2.8, and then further dialyzed against 50 mM Tris, pH 8.2. Concentrations of [^{3}H]folic acid ranging from 1.56 to 50 nM were incubated in duplicate in a single assay for 16 h at 4°C with 100 ng FBP, with and without excess unlabeled folic acid (1 μg), in a total volume of 0.5 ml 50 mM Tris, pH 8.2, for 24 h at 4°C. Bound and free were then separated by adding 0.5 ml 0.25% charcoal·0.025% dextran in 50 mM Tris, pH 8.2, incubating samples for 15 min, and centrifuging samples at 1000 × *g* for 10 min. An aliquot (700 μl) of the resulting supernatant was then subjected to scintillation counting.

Generation of FBP Antiserum and Immunoblotting

FBP from allantoic fluid was used to generate specific antiserum in a rabbit. The rabbit was immunized with 100 μg FBP in 500 μl distilled water mixed with 500 μl complete Freund's adjuvant. The rabbit received two further immunizations at 2-wk intervals with 50 μg FBP in incomplete Freund's adjuvant. Two weeks after the last immunization, blood was collected and allowed to clot, and serum was collected by centrifugation. Antibody specificity to FBP was subsequently tested by immunoblotting. Purified allantoic fluid FBP (10 μg), allantoic fluid proteins, fetal liver membrane proteins, endometrial membrane proteins, and uterine flush proteins were all subjected to SDS-PAGE; the gels were then blotted onto nylon-supported nitrocellulose. Blots were immunostained as described previously [21] except that a 1:1000 dilution of the anti-FBP antiserum or normal rabbit serum was used as first antibody. Also, a 1:2000 dilution of the second antibody (anti-rabbit IgG-horseradish peroxidase) was used in a buffer containing 1% nonfat dry milk and 1% pig serum to decrease nonspecific binding. In addition, 1% nickel sulfate was added to the diaminobenzidine solution to darken the staining reaction.

Intrauterine FBP in Pregnant and Nonpregnant Gilts

To determine whether the presence of FBP changes within the uterus during early pregnancy, white crossbred gilts (1/4 Landrace, 1/4 Yorkshire, 1/4 Chester White, 1/4 Large White) were observed for estrus and were assigned either to be mated or to remain nonpregnant after at least one estrous cycle of normal (17–23 days) length. Gilts (pregnant or nonpregnant) were slaughtered on Days 10, 11, 12, 13, and 15. At slaughter the uterus was recovered, and each uterine horn was flushed with 20 ml Minimum Essential Medium containing one tenth the normal amount of leucine. Pregnancy was confirmed by the presence of conceptuses. Flushings from the two uterine horns of each gilt were pooled, and an aliquot (100 μl) was lyophilized and subjected to SDS-PAGE and immunoblotting using anti-FBP antiserum or normal rabbit serum as described above. To measure folate binding in the same samples, 5 ml of the pooled uterine flushing from each gilt was dialyzed extensively against 50 mM glycine, pH 2.8; this was followed by dialysis against 50 mM Tris, pH 8.2. All samples were diluted 1:20 in Tris and then incubated as described above with 20 nM [^{3}H]folic acid with and without excess (1 μg) unlabeled folic acid. Bound folic acid was separated from free folic acid, and an aliquot of the supernatant was subjected to scintillation counting as described above.

Statistical Analysis

The [^{3}H]folic acid-binding data for the purified allantoic fluid FBP were analyzed using the Ligand (NIH, Bethesda, MD) program to obtain the dissociation constant (K_d) and concentration of receptor site estimates [22]. Data from [^{3}H]folic acid binding of uterine flushings were converted to nanomoles folic acid bound using the known specific activity of the [^{3}H]folic acid used. This concentration was multiplied by the volume of uterine flushings obtained for each gilt to obtain a value for total recoverable folate-binding capacity per uterus. These data were then examined by ANOVA and also by heterogeneity of regression using day as a continuous variable. For ANOVA, the model used included the effects of day and status and the status × day interaction. Heterogeneity of regression tests were performed using a model that included the effect of status, the linear effect of day, the quadratic effect of day, and the interactions of status with the linear and quadratic effects of day. These data were log transformed before analysis to alleviate a scale effect.

RESULTS

Typical chromatograms resulting from purification of FBP from pseudopregnant flushings and allantoic fluid are illustrated in Figure 1. Yield of FBP from pseudopregnant flushings was low (< 1 μg), necessitating the use of silver staining [23] to visualize the resultant protein after SDS-PAGE. A 20 000 M_r band resulted from SDS-PAGE analysis (Fig. 2). The N-terminal sequence of this band was FNWDHXGKMEPAXKRHFXXXTXLYX, which had 72% amino acid sequence identity with bovine milk FBP beginning at amino acid 64 [24], suggesting that the protein was proteolytically degraded. Because of the low yield and the likelihood that the FBP from pseudopregnant pigs was either proteolytically cleaved or otherwise truncated, this protein was not characterized further.

The yield of FBP from allantoic fluid was substantially greater (~300 μg/3 L), and SDS-PAGE followed by Coomassie staining indicated that the protein was homogeneous and had a molecular mass of 30 000 Da (Fig. 2). N-terminal amino acid sequencing indicated the sequence ARAKTDMLNVXMDAKHHKPKPSXED, which had 68%

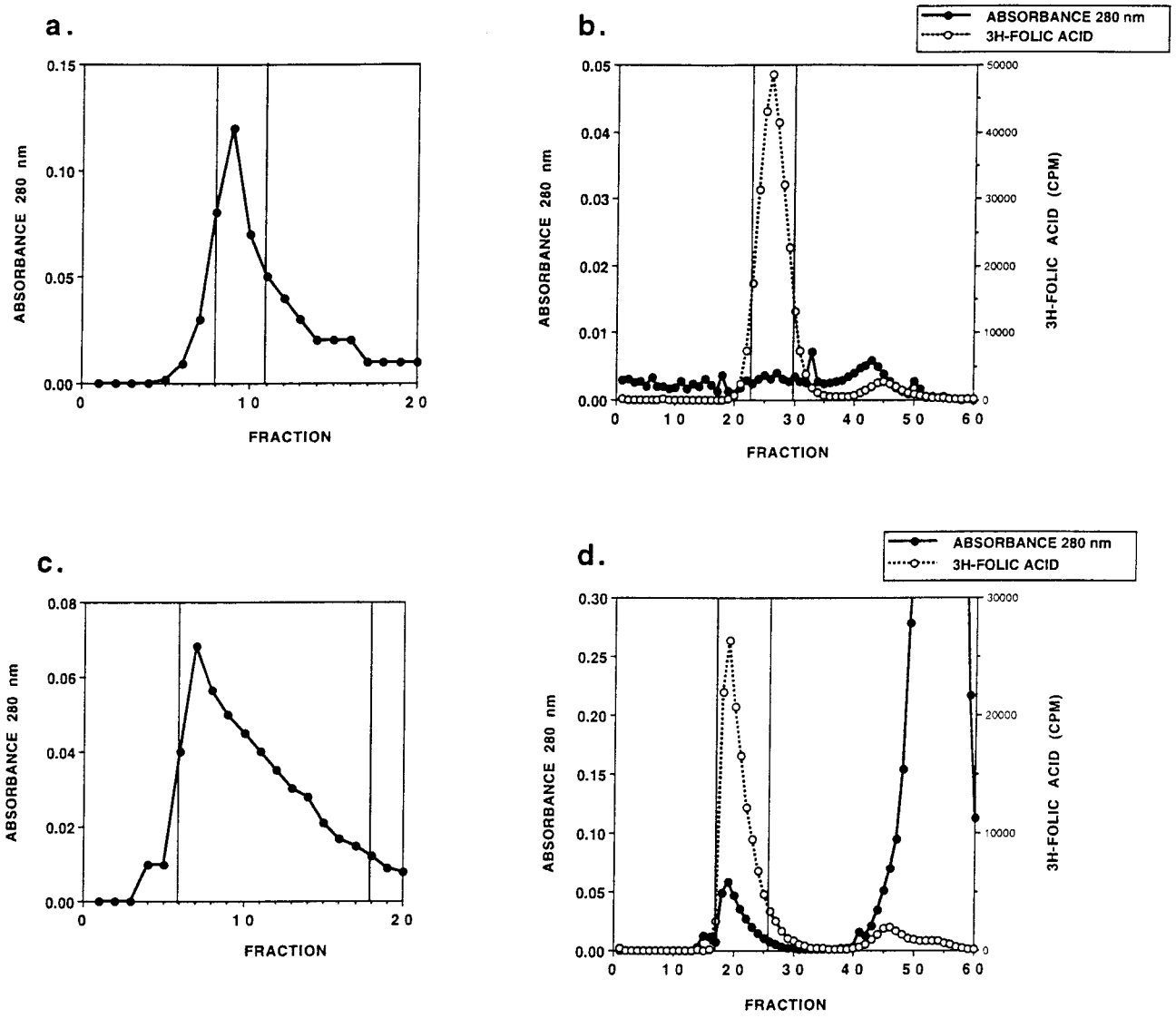

FIG. 1. Representative folate-Sepharose (**a, c**) and G–100 Sephadex (**b, d**) chromatograms from the purification of FBP from Day 60 pseudopregnant uterine flushings (**a, b**) and Day 60 allantoic fluid (**c, d**). Lines indicate the fractions pooled for subsequent steps.

amino acid sequence homology with bovine milk FBP [24] beginning at amino acid 4, suggesting that the protein was substantially intact. Scatchard analysis (Fig. 3) of purified allantoic fluid FBP indicated a K_d of 0.54 nM, and the concentration of binding sites was 1.4 nM. Assuming a molecular mass for FBP of 30 000 Da and a single binding site, only 21% of the FBP actually bound [^{3}H]folic acid. The loss of binding may be the result of the repeated low-pH treatments during purification and processing before Scatchard analysis was done.

The FBP antiserum bound specifically to FBP purified from allantoic fluid. Immunoblotting of allantoic fluid, fetal liver microsomal membrane, and endometrial microsomal membrane proteins resulted in no detectable specific binding (data not shown). Immunoblotting of Day 15 pregnant uterine flush proteins resulted in a specific band of approximately 30 000 M_r (Fig. 4).

Immunoblots of uterine flushings collected from pregnant and nonpregnant gilts on Days 10, 11, 12, 13, and 15 indicated that very little detectable FBP was present on Day 10 of the cycle or pregnancy, and the amount of detectable FBP increased progressively to Day 15 in pregnant and nonpregnant gilts (Fig. 4). Mean recovered volumes of uterine flushings ranged from 36.4 to 46.1 ml and showed no detectable pattern in relation to age; thus immunoblot results reflect intrauterine content of FBP. Least-squares means for total intrauterine folic acid binding are illustrated in Figure 5. Binding increased ~140-fold from Day 10 to Day 15, and this pattern did not differ between pregnant and nonpregnant pigs.

DISCUSSION

This study is the first to describe a folic acid-binding protein in the intrauterine environment in pigs. Results indicate that this protein is similar to other known FBPs from other species [24–28], that it has high affinity for folic acid, and that the amount of the protein present in the intrauterine environment increases from Day 10 to 13 in pregnant and nonpregnant gilts. The pattern of increase of this protein within the uterus suggests that it plays a role in delivering folic acid to the developing conceptus. This period of pregnancy corresponds to a time of rapid growth of the conceptus [29] and initiation of erythropoiesis [30]. Because

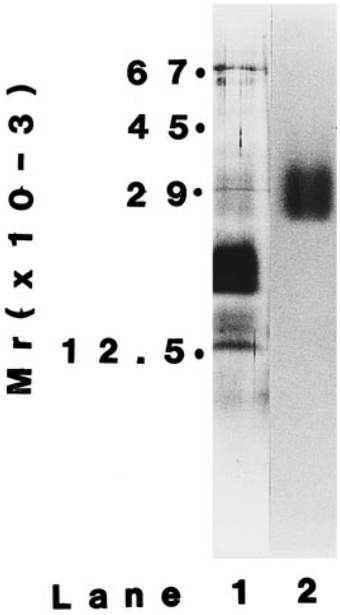

FIG. 2. Representative SDS-PAGE gel of FBP purified from Day 60 pseudopregnant uterine flushings (< 1 μg; lane 1, stained with silver) and Day 60 allantoic fluid (10 μg; lane 2, stained with Coomassie).

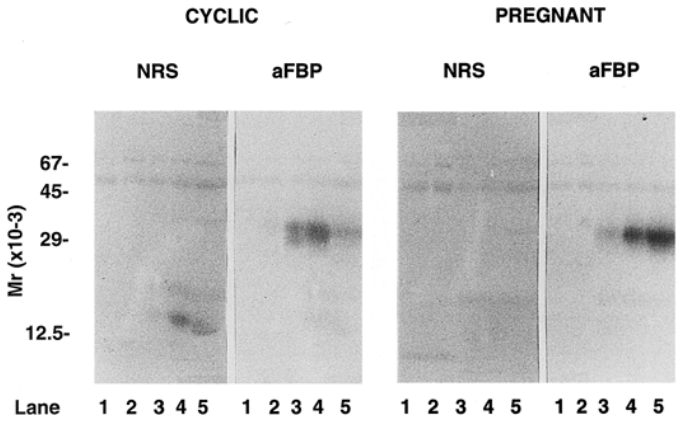

FIG. 4. Representative immunoblots of uterine flushings from nonpregnant and pregnant gilts collected on Days 10, 11, 12, 13, and 15 (lanes 1–5, respectively). Blots were incubated with a 1:1000 dilution of either normal rabbit serum (NRS) or FBP antiserum (aFBP) followed by anti-rabbit IgG second antibody and diaminobenzidine as stain. An aFBP-specific 30 000 M_r band was present on Days 12, 13, and 15 that was not present on Day 10. There were no obvious differences between uterine flush samples collected from pregnant and nonpregnant gilts. The apparent decrease for the Day 15 nonpregnant gilt was not consistent among gilts.

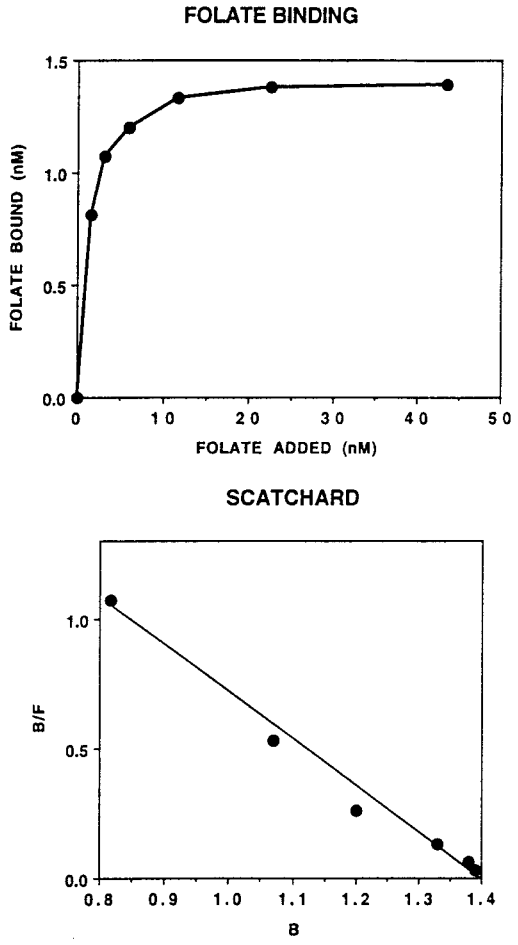

FIG. 3. Saturation curve (**top**) and Scatchard plot (**bottom**) of purified allantoic fluid FBP (100 ng). Scatchard analysis indicated a K_d of 0.54 nM and concentration of binding sites equal to 1.4 nM.

folic acid is a known component of methionine, purine, and thymidine synthesis [1], it is likely that the increase in recoverable FBP reflects an increase in the requirement for folic acid by the conceptus during this period.

The SDS-PAGE analysis of purified FBP indicates a broad diffuse band for both sources. The best explanation of this band pattern is that FBP is probably a glycoprotein.

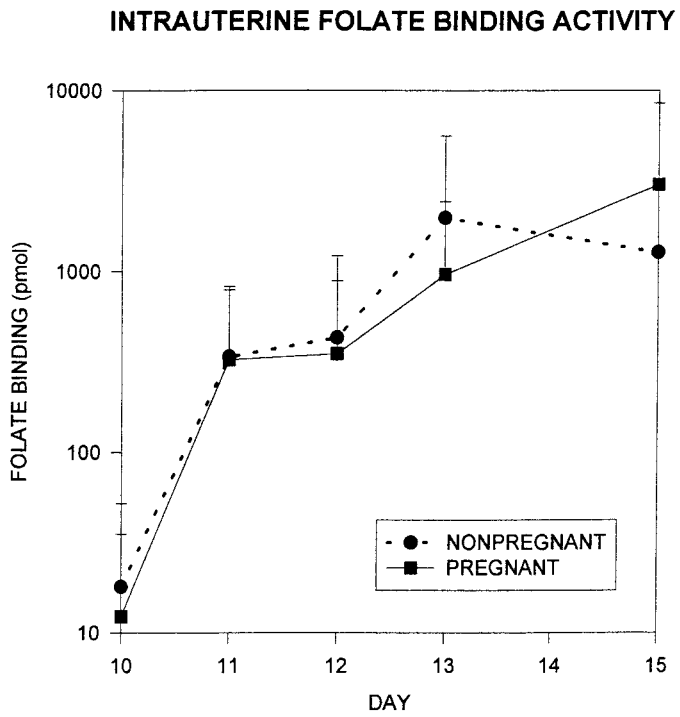

FIG. 5. Least-squares means plus SEM from ANOVA for total folate binding in uterine flushings from pregnant and nonpregnant gilts collected on Days 10, 11, 12, 13, and 15. Analysis indicated no difference between cyclic and pregnant gilts. Note the log scale for folate binding. Number of observations for pregnant and nonpregnant gilts were, respectively, 4 and 4 on Day 10; 5 and 6 on Day 11; 6 and 4 on Day 12; 5 and 4 on Day 13; and 4 and 5 on Day 15.

Analysis of a partial cDNA for FBP [31] indicates several putative *N*-glycosylation sites.

The N-terminal sequence of pig intrauterine and allantoic fluid FBP indicates that these proteins are related to a group of known folate-binding/receptor proteins. Serum of many species is known to contain FBP [32]. FBP are also found in milk [24]; these are thought to aid in the transfer of folate into milk during lactation. Human and mouse folate receptors alpha and beta, which are related to serum and milk FBP [24–28], are glycophosphatidylinositol-anchored membrane proteins thought to be involved in transport of folates into cells [33]. Human folate receptor gamma lacks the glycophosphatidylinositol linkage site and is thought to be a secreted protein, possibly involved in folate transport [28, 34]. Folate receptors can be released from cells by proteolytic cleavage of the protein N-terminal to the glycosylphosphatidylinositol anchor site [35, 36], or by phospholipase C cleavage of the inositol phospholipid to which the protein is attached. This information suggests several potential mechanisms that could be responsible for the increased intrauterine FBP observed in this study, including increased transcription of FBP mRNA, increased translation, increased secretion if the protein is a secreted protein, or increased release from the endometrial cell surface if the protein is membrane bound. Isolation of a full-length cDNA for this protein is currently in progress and will allow investigation of these possible mechanisms of control.

Scatchard analysis suggested that only ~20% of the purified allantoic fluid FBP remained capable of binding folic acid. Because folic acid-Sepharose chromatography was used as a first step in the purification of the protein, all of the protein retained by the column must have been able to bind folic acid at that point. Subsequent elution from the column with low-pH buffer, followed by stripping of folic acid using low-pH buffer in order to do the Scatchard analysis, may have denatured some of the FBP present in the purified preparation. The measurement of affinity resulting from Scatchard analysis could be questioned because it could also be altered by the low-pH treatment. However, the affinity of the purified protein for folic acid was high (~0.5 nM), indicating that the true affinity for folic acid is likely to be at least as high or higher.

Both immunoblot analysis and [^{3}H]folic acid-binding studies indicated that the amount of FBP increased dramatically between Days 10 and 15 of pregnancy. This period of pregnancy is concurrent with increased secretion of other proteins by the uterus, including uteroferrin and retinol-binding protein [37–39]. It is also coincident with blastocyst elongation and the initiation of conceptus estrogen secretion [38]. However, no difference was found in the pattern of change in folic acid binding between pregnant and nonpregnant gilts, suggesting that the conceptus does not influence the amount of FBP that is present within the intrauterine environment. Thus it seems unlikely that the increase in FBP is a response to conceptus estrogen secretion. Furthermore, because the conceptus does not influence folic acid binding, the conceptus is an unlikely source of this protein. The most likely source of the FBP is either serum transudate or endometrium. Attempts to demonstrate synthesis of the protein by endometrium in culture, using [^{3}H]leucine to label endometrial products along with immunoprecipitation analysis, have been unsuccessful (unpublished results). The medium used (Minimum Essential Medium) contained very high folate concentrations, and it is possible that this may have interfered with FBP synthesis. Alternatively, the level of secretion of this protein may be too low to be detectable using [^{3}H]leucine and immunoprecipitation analysis. Finally, because the protein may be a membrane protein, conditions for release of the protein from the cell membrane may not be met in culture (proteases or phospholipases secreted by the endometrium that might normally perform this function would be highly diluted in culture). Isolation of the cDNA for FBP, along with further information regarding how intrauterine concentrations of this protein are controlled, may provide further insight into its source and control of its secretion.

It was surprising that FBP could not be detected in allantoic fluid by immunoblotting. Our best explanation is that the concentration of FBP in allantoic fluid is below the detection limit of our immunoblotting method. Subsequent measurements using RIA (unpublished results) indicate that FBP concentrations in allantoic fluid are much lower than in uterine flushings.

The N-terminal sequencing of FBP from pseudopregnant uterine flushings clearly indicated that proteolytic cleavage of the protein had occurred, and this may explain the low yields of FBP obtained from that source. The most likely explanation is that FBP is a substrate for one or more proteases within the intrauterine environment. Proteolytic degradation of FBP within the pseudopregnant uterus may be a way of retrieving folic acid that has been secreted by the endometrium and is not used because of the lack of a conceptus. Because the interactions between FBP, possible proteases, and protease inhibitors are likely to influence folate transport within the intrauterine environment, these interactions warrant further investigation.

In conclusion, in this study FBP from Day 60 porcine allantoic fluid was purified. Specific antiserum to this protein was generated, which should be useful for further studies using immunoblotting and for the development of an RIA for this protein. The FBP has a molecular mass of 30 000 Da and a K_d for folic acid in the subnanomolar range. Immunoblotting and folic acid-binding studies indicate that the amount of FBP increases dramatically within the intrauterine environment between Days 10 and 15 in both pregnant and nonpregnant gilts. These data are consistent with a role for FBP in the transport of folic acid to the developing conceptus.

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